RESPONSE AND REMARKS

I. Status of the Claims

Claims 1 - 20 are pending in the application.

Pursuant to a Restriction Requirement, Applicants provisionally elected, with traverse, the claims of Group II. As a result, claims 1 and 10 – 20 are currently withdrawn from consideration in this application.

Claims 2 – 9 are pending, of which claim 2 is the sole independent claim.

Claims 2 – 9 are rejected under 35 U.S.C. § 112, second paragraph, as being indefinite, and are rejected under 35 U.S.C. § 103 for obviousness.

By the foregoing "Amendments to the Claims," claims 2, 3, 4, 7, and 8 are amended as discussed below.

II. Amendments to the Claims

In response to the referenced Office Action and for clarity, claims 2, 3, 4, 7, and 8 have been amended as shown above on pages 2 and 3 hereof. The amendments to the claims are supported by the specification and claims of the application as filed.

Claim 2 has been amended to add the phrase "in an amount" to the step of adding spores for the sole purpose of making the step more grammatically clear. Claim 2 has also been amended by adding the steps of "incubating" and "recovering." Applicants agree with the Examiner that there is no rule or other requirement for an express step of recovery, especially in light of the level of skill in the art and the disclosures in the specification. The addition of these steps makes the claimed method explicitly complete, rather than implicitly complete as originally presented, and does not alter the scope of the claim. All of the terms in this claim are adequately defined in the art and/or the specification.

Claim 3 has been amended to add reference to "the step of adding" in claim 2, thereby providing a proper antecedent reference. Applicants submit that claim 3 as previously presented

was technically and grammatical clear, but because the amendment does not affect the scope of the claim the amendment is submitted to present a more traditional expression.

Claim 4 has been amended to correct the erroneous lack of punctuation.

Claim 7 has been amended to refer simply to a ratio of 1:1. This amendment makes the claim clear and does not alter the scope of the claim as originally presented.

Claim 8 has been amended by adding reference to "use" of a filter and to clarify that the measurement refers to the pore size of the filter. While these amendments simply clarify what is expressly stated in the specification, the additions make the claim more complete. The amendment does not affect the scope of the claim.

III. Response to the Office Action

Applicants appreciate the Examiner's explanation of the disposition of the claims, but respectfully request that, in light of the foregoing amendments and the following considerations, the Examiner reconsider and withdraw the rejections stated in the Office Action. Applicants respectfully submit that, in light of the nature of the art with which the invention is concerned, there has been an insufficient showing of a teaching, suggestion, or motivation to combine the cited references so as to establish that the claimed invention would have been obvious. Moreover, there is no showing that a person of ordinary skill in the art would have had a reasonable expectation of the success in the result of combining the teachings of the references.

Applicants submit that the rejections under § 112 have been obviated by the amendments entered above. Applicants believe that the claims as originally presented do particularly point out and distinctly claim the subject matter of the invention when viewed by one of ordinary skill in the art who is aware of Applicants' specification. While Applicants agree with Examiner that there is no statute or regulation requiring positive recitation of recovery step in claim 2, the addition thereof does not alter the scope of the original claim and aids completeness. As to the step of "incubating," Applicants submit that one of skill in the art, especially as taught by Applicants' specification, would know what the previous step of "adding" would entail in this art. Again, the addition of this step does not alter the scope of the claim and is included for completeness.

Claim 3 has been amended to add language clearly pointing out the antecedent, and claim 4 has been amended to correct the lack of punctuation.

Claim 7 has been amended to reflect a ratio of about 1:1, with extraneous language being removed. Claim 8 has been amended grammatically and for clarification.

All of the foregoing amendments are supported by the specification and claims as originally filed. Applicants submit that the claims as amended comply with § 112, second paragraph, and respectfully request that the rejections thereunder be withdrawn.

Claims 2 – 9 have been rejected under § 103(a) as being unpatentable over Ireland *et al.* (*Infection and Immunity*, 70:10, pp. 5870-5872 (2002))(hereafter "Ireland") taken with ATCC Catalogue and Citri (U.S. Patent No. 5,614,375)(hereafter "Citri"). As set forth in the Office Action, the rejection relies on a teaching by Ireland of making a germinant by adding *B. anthracis* endospores to RAW264.7 cells, the asserted fact that the IC-21 cell line is "old and well know[n] in the art," and the teaching of Citri that germinants are known for *Bacillus* strains.

Applicants respectfully submit that the reasoning in the Office Action does not establish a *prima facie* case of obviousness. The U.S. Circuit Court of Appeals for the Federal Circuit has often stated the burden and the standard regarding obviousness:

> The PTO has the burden under section 103 to establish a *prima facie* case of obviousness. It can satisfy this burden only by showing some *objective* teaching *in the prior art* or that *knowledge generally available* to one of ordinary skill in the art would lead that individual to combine the relevant teachings of the references.

In re Fine, 837 F.2d 1071, 1074 (Fed.Cir. 1988)(emphasis added)(internal citations omitted). The Court has also stated, with respect to obviousness, that: "[A] proper analysis under § 103 requires, inter alia, consideration of two factors: 1) whether the prior art would have suggested to those of ordinary skill in the art that they should make the claimed composition or device, or carry out the claimed process; and 2) whether the prior art would have also revealed that in so making or carrying out, those of ordinary skill in the art would have a reasonable expectation of success." *In re Vaeck*, 947 F.2d 488, 493 (Fed.Cir. 1991), *cited in Noelle v. Lederman*, 355 F.3d 1343, 1348 (Fed.Cir. 2004).

The reasoning proffered in the Office Action does not satisfy the requirements that the USPTO establish objective teachings leading a person of ordinary skill in the art to combine the

cited references, nor does it satisfy the requirement that there be a reasonable expectation of success in the combination. These teachings must be found either in the prior art itself or in the knowledge generally available to one skilled in the art.

Applicants respectfully submit that the Office Action has failed to set forth an objective teaching in the prior art that would lead one of skill in the art to Applicants' claimed invention. The teaching of Ireland is *not* a process of making a germinant by providing macrophages and adding spores. The teaching of Ireland is that, under conditions that Ireland carefully and precisely defines, adding certain spores to the RAW264.7 macrophage will produce what appears to be a germinant. Ireland says nothing about macrophages in general, and specifically says nothing about IC-21.

The mere fact that the ATCC Catalogue indicates that IC-21 is "old and well know[n] in the art" teaches nothing about the production of germinants. There is no statement or reference in the catalogue listing that would suggest that a germinant could be produced by exposing IC-21 cells to spores, and there is no statement that IC-21 would react to spores in a manner similar to that of RAW264.7. Citri's teaching that chemical germinants such as glucose are known does not teach or suggest that macrophages, and particularly IC-21 would produce an effective germinant.

It is not sufficient, in the absence of an objective teaching, to merely substitute one component or step in a reference for a component or step in another reference. *See, e.g., In re Fine, id.* at 1074-1075. Simply substituting IC-21 for Ireland's RAW264.7 has no basis in the art. "'[O]bvious to try' is not a legitimate test of patentability." *In re Geiger*, 815 F.2d 686, 688 (Fed.Cir. 1987).

Using the claimed invention as a blueprint to tailor a combination of prior art, of course, is impermissible hindsight, *W.L. Gore & Assoc. v. Garlock, Inc.*, 721 F.2d 1540, 1553 (Fed.Cir. 1983), and cannot support a *prima facie* case of obviousness. The cited art does not suggest any teaching broadly applicable to *all* macrophages, and sets forth no teachings about IC-21 in particular.

Not only does the prior art not teach that it would have been obvious from Ireland, with or without other references, to substitute the IC-21 of Applicants' claimed invention for the RAW264.7, the art teaches away from such a substitution. Applicants' work, set forth in the

specification and drawings, indicates that under the conditions used by Applicants (which does produce a germinant), the cell lines RAW264.7 and J774A.1 both fail to produce germinants. Nothing in the Office Action indicates that Applicants' findings or data are incorrect. Further evidence of the differences among these cell lines is shown in published literature, an example of which is attached hereto for the Examiner's convenience. In the attached journal article from *Toxicology in Vitro*, **19** (2005) pp. 221-229, experimental data are set forth regarding the effects of *B. anthracis* (BA) on different macrophages. Differences among the tested macrophages are noted throughout the article, a clear example of which is shown at Fig. 2 and accompanying text (*id.*, at p. 225). Upon exposure to BA over a fifteen hour period, the macrophages IC-21, RAW264.7, and J774A.1 showed markedly different reactions. No significant mortality was found in the IC-21, whereas RAW264.7 showed an 80% mortality at 7.5 hours and J774A.1 showed a 50% mortality at the time. At 15 hours, IC-21 exhibited little or no mortality, whereas both of the others exhibited 100% mortality.

The article, and Applicants' specification, are objective evidence that those of skill in the art would not expect macrophages to react similarly in the presence of endospores. Rather than suggesting that IC-21 would react similarly to RAW264.7, the objective evidence suggests the opposite.

Applicants respectfully submit that the USPTO has failed to establish a *prima facie* case of obviousness. There is no objective teaching that would lead one of skill in this art to selectively pick the macrophage selected by Applicants, nor is there an objective teaching that, once selected, the selected IC-21 would respond as did the RAW264.7. As shown, the art itself teaches against such any reasonable expectation of success. Because it is impermissible to use an "obvious to try" standard, or to use Applicants' invention as a blueprint for selecting specific components, there is no showing that Applicants' invention would have been obvious at the time it was made.

IV. Conclusion

Based on the foregoing, Applicants submit that the claims are currently in compliance with § 112 and are not invalid under § 103. Applicants request that the rejections of the pending claims be withdrawn, and that the claims be found to be in condition for allowance. Early notice of allowability is respectfully solicited.

Attorney Docket No.: Navy Case 84554

Should the Examiner have any questions about this application or Response or believe that discussion would advance the prosecution of this application, the Examiner is invited to contact Applicant's representative at the telephone number listed below.

Respectfully submitted,

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Differential susceptibility of macrophage cell lines to *Bacillus anthracis*–Vollum 1B

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Abstract

Bacillus anthracis (BA) is a spore forming bacterium and the causative agent of anthrax disease. Macrophages (Mϕs) play a central role in anthrax disease. An important step in disease progression is the ability of BA to secrete lethal toxin (LeTx) that kills Mϕs. LeTx is a heterodimer composed of protective antigen (PA) and lethal factor (LF). Researchers have shown that Mϕ cell lines demonstrate differential susceptibility to purified LeTx; for example RAW264.7 and J774A.1 Mϕs are sensitive to LeTx whereas IC-21 Mϕs are resistant. Research has also suggested that exogenous factors, including other BA proteins, can influence the activity of LeTx. For this reason, the objective of the current work was to examine if RAW264.7, J774A.1, and IC-21 Mϕs demonstrated differential susceptibility when cultured with a LeTx-producing strain of BA. Here, we co-cultured Mϕs with LeTx$^+$ Vollum 1B (V1B) spores for >15 h and assayed for Mϕ cell death by morphology, trypan blue (TB) staining, neutral red (NR) activity, and lactate dehydrogenase (LDH) activity in the culture media. Following the addition of V1B spores, necrosis (≈50% mortality) was observed in RAW264.7 and J774A.1 Mϕs at 7.5 and 10 h, respectively. By 15 h, both RAW264.7 and J774A.1 Mϕs demonstrated 100% mortality. In contrast, IC-21 Mϕs, under identical culture conditions, remained viable (98%) and activated throughout the course of the experiment (>24 h). The mechanism of RAW264.7 cell death appeared to involve LeTx because the V1B-induced cytotoxicity was dose-dependently reversed by the addition of anti-PA antibody to the culture media. These observations suggest there is differential susceptibility of Mϕ cell lines to the LeTx$^+$ V1B strain of BA. Further development of this in vitro model may be useful to further characterize the interactions between Mϕs and BA spores.
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Keywords: Macrophage; Vollum 1B; Anthrax; Toxicity; Lethal factor

1. Introduction

Bacillus anthracis (BA) is a large, gram-positive, spore-forming, non-motile bacterium that is the causative agent of anthrax disease (Takamatsu and Watabe, 2002). BA spores are the infective form of the bacteria and are characterized as dehydrated particles that lack measurable metabolic activity (Inglesby et al., 2002). Following infection, BA spores germinate into vegetative cells that are metabolically active and secrete several known toxins. Generally, clinical manifestations of anthrax disease reflect the bacteria's route of entry-cutaneous, gastrointestinal and inhalation, with more than 95% of natural occurring cases cutaneous (Dixon et al., 1999). However, there is a renewed interest in

Abbreviations: BA, *Bacillus anthracis*; LDH, lactate dehydrogenase; Mϕ, macrophage; NR, neutral red; TB, trypan blue; V1B, Vollum 1B; LeTx, lethal toxin; EdTx, edema toxin

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the inhalational form of anthrax disease due to the potential to use BA as an airborne biological weapon and a mortality rate approaching 100% (Matsumoto, 2003; Henry, 2001).

Macrophages (Mϕs) play key roles in inhalational anthrax. First, following deposition in the lungs, BA spores are phagocytosed by alveolar Mϕs and transported to the draining mediastinal and treacheobronchial lymph nodes (Henry, 2001; Shafa et al., 1966). Second, it is thought that BA germinates from its dormant spore state into its metabolically active vegetative state inside the Mϕ during migration (reviewed in Guidi-Rontani, 2002). Germination is closely followed by synthesis of several virulence factors that are required for disease progression (Guidi-Rontani et al., 2001; Dixon et al., 2000; Guidi-Rontani et al., 1999). Finally, the late stage of systemic anthrax disease is characterized by sudden shock caused by TNF-α and IL-1 secreted, in part, from Mϕs (Dixon et al., 1999).

The virulence of BA is due to three known factors: lethal toxin (LeTx), edema toxin (EdTx), and an antiphagocytic capsule (Ascenzi et al., 2002). Like many bacterial toxins, LeTx and EdTx each possess two subunits, a B-domain (cell binding) and an A-domain (active or enzymatic). The A-domain for LeTx is a 90 kDa protein called lethal factor (LF), which is a highly specific metalloprotease that cleaves mitogen-activated protein kinase kinase (MAPKK) leading to interruption of cellular signaling pathways in susceptible cells (Pannifer et al., 2001; Hanna, 1999). The A-domain for EdTx is an 89 kDa protein called edema factor (EF), which is a calmodulin-dependent adenylate cyclase that interferes with cellular signaling events by increasing levels of cyclic AMP (cAMP) in susceptible cells (Kumar et al., 2002; Leppla, 1982). The B-domain for both LeTx and EdTx is an 83 kDa protein called protective antigen (PA), which binds cell surfaces and delivers either LF or EF to the cytosol. LF, EF and PA are non-toxic individually and are coded by separate genes located on a single plasmid [pXO1; 184.5 kilobase]. The genes for the third virulence factor, an antiphagocytic capsule, are coded on a separate plasmid [pXO2; 95.3 kilobase] and are involved in synthesis of a poly-D-glutamic acid capsule that inhibits phagocytosis of vegetative BA (Makino et al., 2002).

LeTx is considered the major virulence factor in BA. Indeed, anthrax disease can be mimicked in animals by exposure to purified LeTx (Hanna et al., 1993). LeTx is also highly cytotoxic for Mϕs and can induce necrosis and apoptosis (Popov et al., 2002; Parker et al., 2002; Hanna et al., 1992). Additionally, Mϕ cell lines, as well as primary cultures, demonstrate differential susceptibility to purified LeTx (Friedlander et al., 1993; Singh et al., 1989). Specifically, RAW264.7 and J774A.1 cell lines are sensitive to LeTx whereas IC-21 Mϕs appear resistant. The mechanisms of action of LeTx in susceptible Mϕs is complex and remains to be clearly defined; however, studies suggest toxicity may involve disruption of second messenger systems (Bhatnagar et al., 1999; Vitale et al., 1998), cytokine production (Kim et al., 2003), proteasome activity (Tang and Leppla, 1999), and/or oxidative stress (Hanna et al., 1994).

Although studies that use purified LeTx and sensitive or resistant Mϕs advance our understanding of anthrax disease, it is important to examine the more complex interactions between Mϕs and live LeTx-producing bacteria for several reasons. For example, other proteins expressed by the bacteria may affect the regulation and activity of LeTx. Indeed, it has been shown that EdTx acts as an enhancer that increases the activity of LeTx in Mϕs (Kumar et al., 2002; Pezard et al., 1991). Thus, studies that examine the affect of purified LeTx on Mϕs may not fully describe the activity of LeTx from bacteria that are concomitantly synthesizing EdTx and/or other proteins. Additionally, BA spores germinate and escape destruction by the Mϕ following phagocytosis, which is thought to be a critical step in the initial stages of infection. The mechanisms used by BA during escape remain to be fully defined but may be closely linked with LeTx gene activation inside the Mϕ (Guidi-Rontani et al., 1999) as well as activation of other undefined 'escape' genes on pXO1 (Dixon et al., 2000). Therefore, examination of Mϕ sensitivity or resistance to purified LeTx cannot address this critical aspect of anthrax disease; live $pXO1^+$ bacteria are required. Finally, it is widely recognized that another important step in anthrax progression involves pro-inflammatory cytokine production from Mϕs and studies suggest that the ability of Mϕs to produce TNF-α in response to LeTx depends on whether LeTx is used in purified form (Erwin et al., 2001) or generated from phagocytosed $LeTx^+$ BA spores (Pickering and Merkel, 2004). Therefore, collectively, examining sensitivity and resistance of Mϕ cell lines to $LeTx^+$ BA spores will not only help to define the actions of LeTx in a more physiologically relevant context (i.e., derived from a live bacteria), but may also help to define the role of other bacterial toxins that can act alone or synergistically with LeTx.

The objective of the present work was to examine the susceptibility of Mϕ cell lines to the Vollum 1B (V1B) strain of BA. V1B has both pXO1 and pXO2 plasmids and therefore contains all known virulence factors ($LeTx^+$, $EdTx^+$, and $capsule^+$). Our experimental design consisted of exposing equal numbers of Mϕs and V1B spores, then measuring Mϕ death over time (based on Guidi-Rontani et al., 2001, 1999). Cytotoxicity was determined using Mϕ morphology, trypan blue (TB) staining, neutral red (NR) activity, and lactate dehydrogenase (LDH) activity. Finally, to begin addressing the mechanism of V1B-induced Mϕ death, the activity of PA was blocked by the addition of anti-PA monoclonal antibody to the cultures.

2. Materials and methods

2.1. Macrophage cells

IC-21 macrophages (TIB-186), RAW264.7 monocyte-macrophages (TIB-71), and J774A.1 monocyte-macrophages (TIB-67) were obtained from American Type Culture Collection (ATCC, Manassas, VA). All cell lines were maintained in growth media and cultured in a tissue culture incubator (37 °C, 100% humidity, 95% air/5% CO_2). Growth media for all macrophages was basal media supplemented with fetal bovine serum (FBS), 10% final concentration (ATCC, catalog # 30-2020) and penicillin–streptomycin (Pen/Strep), 1% final concentration (Vitacell, ATCC, catalog # 30-2300). Basal media for IC-21 and RAW264.7 Mϕs was RPMI 1640 (Vitacell RPMI-1640 media, ATCC, catalog # 30-2001) and basal media for J774A.1 Mϕs was Dulbecco's Modified Eagle's Media (Vitacell DMEM media, ATCC, catalog # 30-200). RAW264.7 and J774A.1 Mϕs were subcultured by scraping and IC-21 Mϕs were subcultured by incubating cells in Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate buffered saline (JRH Bioscience, catalog # 59321-78P, Lenexa, KS). Subculture ratio for all cell lines ranged from 1:2 to 1:4 and were subcultured less than 20 times. In all experiments, cell numbers were determined using a hemacytometer and viability was determined using trypan blue staining, which always exceeded 95%. Anti-PA antibody (catalog # C86601M) was purchased from BioDesign International (Saco, ME).

2.2. Bacillus anthracis (V1B)

V1B ($pXO1^+$, and $pXO2^+$) was kindly provided by Michael Chute (Naval Medical Research Center, Bethesda, MD). In all experiments, V1B was quantified as colony forming units (CFU) by dilution plating on Trypticase Soy Agar with 5% Sheep blood plates (BD Bioscience). V1B spores were produced in modified Schaeffer's sporulation media (Leighton and Doi, 1971) and prepared using published protocols (Dixon et al., 2000; Guidi-Rontani et al., 1999).

2.3. Culture conditions

The culture conditions used in the present work was based on previous experiments that demonstrated strain differences in intracellular accumulation of *B. anthracis* during infection of RAW264.7 Mϕs (Dixon et al., 2000). In that previous study, differential responses of *B. anthracis* strains were determined after incubating RAW264.7 Mϕs with an MOI (multiple of infection) of 10 (10 spores:1 Mϕ) for 30 min to allow for phagocytosis followed by washing to remove non-phagocytosed spores. In the present work, Mϕs (1×10^5) were seeded in a single well of a 24-well tissue culture plate using 500 μl growth media for 1–2 h. Thereafter, macrophages were washed one time with basal media and then 500 μl of the respective basal media was placed in the well. V1B spores (1.5×10^6 CFU in 20 μl 0.1X PBS; MOI = 15) or 20 μl 0.1X PBS-only control were immediately added to the culture and allowed to incubate for 30 min (37 °C, 95% air/5% CO_2). After incubation, the culture supernatant containing non-phagocytosed spores was removed; the wells were washed twice with sterile PBS, followed by one wash with basal media, and then 500 μl basal media was placed in the well and the culture was returned to the incubator (time = 0 h).

2.4. Cytotoxicity assays

Mϕ cytotoxicity assays included morphology, trypan blue (TB) dye exclusion, neutral red (NR) uptake, and lactate dehydrogenase (LDH) activity in the culture supernatant. For TB tests, the culture supernatant was removed and replaced with 500 μl trypan blue solution (0.4%, Sigma, catalog # T18-54) that was diluted 5-fold using sterile phosphate buffered saline (PBS) at the indicated time point. Mϕs were immediately examined for TB uptake using a Zeiss Axiovert inverted microscope. Here, photographs (40X) were taken of each Mϕ-V1B culture, alternating between treatment and control wells. One photograph was taken of each culture well ($n = 4$ per experiment for all treatment and control groups) and the TB data presented in the current work (Fig. 2) was pooled from two separate experiments (for a total of $n = 8$ for all groups). The percent viability was determined by counting individual live and dead cells in the photographs (see Fig. 1 for representative photographs). For TB dye exclusion, dead Mϕs stained blue and live Mϕs excluded the dye remaining clear. For NR uptake, the culture supernatant was removed two hours before the indicated time point and replaced with fresh basal media supplemented with 5% NR solution (Sigma, catalog # N2889) that was filter-sterilized (0.2 μm). The cultures were then incubated for an additional 2 h (100% humidity, 95% air/5% CO_2). Thereafter, the culture supernatant was removed, the cells were washed once using sterile PBS, washed once with fixative (0.1% $CaCl_2$ in 0.5% formaldehyde), and then the incorporated dye was solubilized in a 1% acetic acid in 50% ethanol solution. The amount of dye was determined by measuring the absorbance at 540 nm. For NR activity, live Mϕs uptake the dye and thus stained red, whereas dead Mϕs are unable to incorporate the dye. For LDH activity in the culture supernatant, the supernatant was removed at the indicated time point and centrifuged at $10{,}000 \times g$ for 10 min (4 °C) to pellet cellular debris. 0.5 ml pyruvate (0.17 g/l in 1 M EPPS buffer, pH = 7.3; Sigma P5280) and 0.5 ml NADH (2 mg/ml in 1 M MOPS buffer, pH = 7.0; Sigma N8129) were mixed in a glass tube

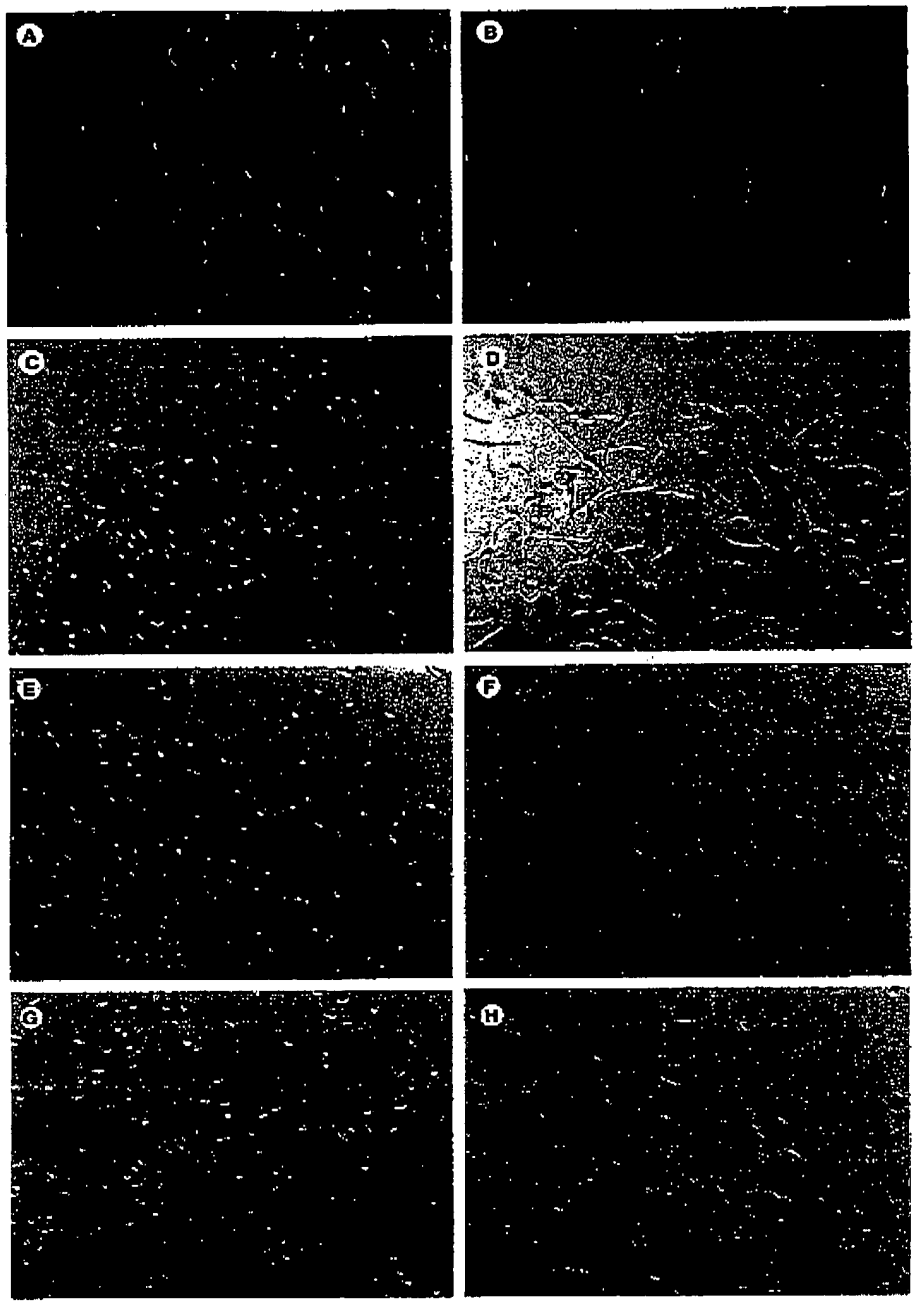

Fig. 1. Representative photographs demonstrating the morphology and viability of IC-21, RAW264.7, and J774A.1 Mϕs co-cultured with V1B. Mϕs were cultured in media alone (A, C, E and G) or with V1B (B, D, F and H). At 15h, V1B had germinated and grown extensively in the presence of IC-21 Mϕs such that the extensive outgrowth of V1B vegetative bacteria is responsible for the cloudiness of photograph B compared to A. No staining with TB was observed in IC-21 Mϕs after 15h of co-culture with V1B (D compared to C). In contrast, RAW264.7 Mϕs co-cultured with V1B, compared to resting RAW264.7 Mϕs, demonstrated significant TB staining after 7.5h (F compared to E), as did J774A.1 Mϕs at 10h (H compared to G). Note, the cultures in photographs C–H were washed one time to allow incubation with TB dye, whereas cultures in photographs A and B were unwashed at the indicated time point (photographs taken at 40X).

and warmed to 37 °C. Thereafter, 450 μl culture supernatant was added to the pyruvate/NADH tube and incubated for 30 min (37 °C). Next, 1.0 ml color reagent (2,4-dinitrophenylhydrazine; 200 mg/l in 1 N HCl; Aldrich D199303) was added and the mixture was incubated at room temperature for 20 min. The reaction was stopped by adding 10 ml of 0.4 N NaOH and incubating for 10 min at room temperature. Thereafter, the absorbance of the resulting hydrazone was determined at 500 nm. For LDH activity, there is an inverse correlation between the amount of LDH in the media and the absorbance of the resulting hydrazone. All statistical analysis on LDH data was conducted on raw data and all data are presented as the inverse of the absorbance (500 nm).

2.5. Statistical analysis

All data are presented as mean ± SEM. Differences between two group means was determined using Student's *t* test and differences among multiple group means were determined using one-way ANOVA with Fisher's least significant difference (LSD) test for making justified pairwise comparisons with a p value of <0.05 considered significant. All statistical analysis was conducted on raw data.

3. Results

3.1. Morphology

When co-cultured with V1B spores, all three Mϕ cell lines demonstrated signs of activation based on morphology. Viable IC-21, RAW264.7 and J774A.1 Mϕs (Fig. 1D, F, and H, respectively) had extensive cellular extensions that suggest these cells are activated and were migrating or phagocytosing bacterial components. Extensive cellular extensions were not observed in control IC-21, RAW264.7 and J774A.1 Mϕs at any time point tested (Fig. 1C, E, and G, respectively). For IC-21 Mϕs, cellular extensions could be seen prior to washing off vegetative V1B in the culture, which has grown to such a degree that it was difficult to focus on the adherent Mϕs on the bottom of the culture well (Fig. 1B treatment versus Fig. 1A control).

3.2. Trypan blue (TB) dye exclusion

Mϕ viability was examined at the indicated time points (0–15 h in culture) using TB dye exclusion. Here, dead and dying Mϕs stain blue, whereas live Mϕs extrude the dye and remain clear. As shown (Fig. 2), there appeared to be differential susceptibility of Mϕ cell lines to V1B. Approximately 80% of RAW264.7 Mϕs co-cultured with V1B appeared dead by 7.5 h and by 15 h

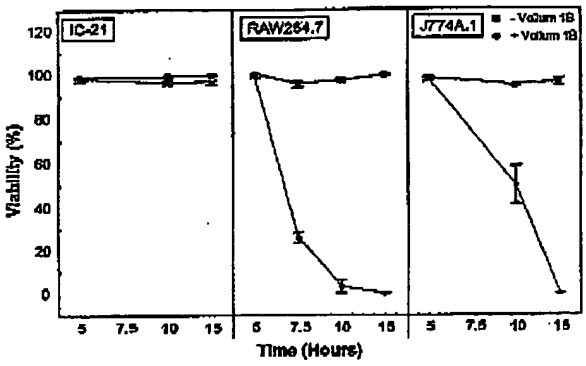

Fig. 2. TB staining of IC-21, RAW264.7, and J774A.1 Mϕs co-cultured with V1B. Mϕs were cultured in media alone or with V1B. At 15 h, no significant staining in TB was observed in IC-21 Mϕs compared to control IC-21 Mϕs. In contrast, a significant increase in RAW264.7 Mϕs and J774A.1 Mϕs staining was observed beginning at 7.5 and 10 h, respectively. The values are mean ± SEM ($n = 8$ for all groups) using data pooled from two separate experiments.

RAW264.7 Mϕs demonstrated 100% mortality. Likewise, approximately 50% of J774A.1 Mϕs co-cultured with V1B stained blue by 10 h and by 15 h J774A.1 Mϕs demonstrated 100% mortality. In contrast, IC-21 Mϕs remained 100% viable throughout the time course of the experiment (15 h). Representative photographs used to collect the data presented in Fig. 2 are shown in Fig. 1C–H.

3.3. Neutral red (NR) uptake

Cellular viability was examined using NR uptake. In this assay, NR is taken up by Mϕs using an active energy-dependent process. Thus, live Mϕs uptake the dye, whereas dead and dying Mϕs do not. RAW264.7 Mϕs co-cultured with V1B demonstrated increased NR activity compared to RAW264.7 controls at 5 h (Fig. 3B). However, at 7.5, 10 and 15 h RAW264.7 Mϕs co-cultured with V1B demonstrated a significant, time-dependent decrease in NR activity compared with controls (Fig. 3B). Similar to RAW264.7 Mϕs, J774A.1 Mϕs co-cultured with V1B demonstrated a significant, time-dependent decrease in NR activity at 10 and 15 h compared to J774A.1 Mϕs (Fig. 3C). In contrast to both RAW264.7 and J774A.1 Mϕs, IC-21 Mϕs co-cultured with V1B appeared to have a significant increase in NR uptake when directly compared to IC-21 controls throughout the course of the experiment (Fig. 3A).

3.4. Lactate dehydrogenase (LDH) activity

LDH activity in the culture supernatant of treatment and control Mϕs was determined throughout the course of the experiment (Table 1). RAW264.7 Mϕs co-cultured

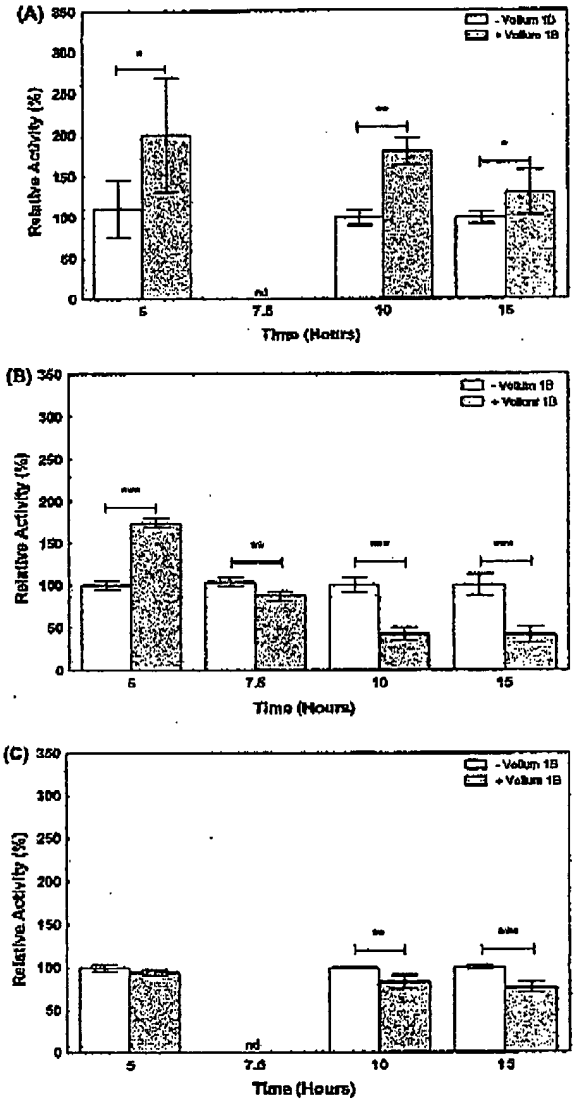

Fig. 3. NR activity in IC-21, RAW264.7 and J774A.1 Mϕs co-cultured with V1B. Mϕs were cultured in media alone or with V1B. NR activity was determined at the indicated time points. An increase in NR activity was detected in IC-21 Mϕs co-cultured with V1B at all time points tested (A). In contrast, RAW264.7 Mϕs co-cultured with V1B had an increase in NR activity at 5h, but activity decreased thereafter (B). J774A.1 Mϕs co-cultured with V1B demonstrated a steady decrease in NR activity from 5 to 15h (C). The values are mean ± SEM ($n = 4$–8 for all groups) and have been reproduced in independent experiments, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$ (nd = not determined).

with V1B appeared to have elevated levels of LDH in the culture supernatant beginning at 7.5h. J774A.1 Mϕs appeared to have elevated LDH level in the culture at 15h only. Finally, LDH activity appeared increased in IC-21 treatment Mϕs at 10 and 15h.

Table 1
Summary of LDH activity in the culture media of IC-21, RAW264.7, or J774A.1 macrophages co-cultured with V1B

	LDH activity in culture media (h)			
	5	7.5	10	15
IC-21	2.10 ± .060	nd	1.80 ± .053	1.74 ± .020
IC-21 + V1B	2.00 ± .080	nd	2.05 ± .052*	2.20 ± .011*
RAW264.7	2.05 ± .050	2.13 ± .063	2.02 ± .030	1.96 ± .146
RAW264.7 + V1B	2.03 ± .050	3.28 ± .360*	2.80 ± .030*	2.93 ± .146*
J774A.1	1.91 ± .070	nd	1.94 ± .087	1.69 ± .031
J774A.1 + V1B	2.00 ± .240	nd	1.91 ± .051	2.07 ± .061*

Note: Data are presented as the inverse of the raw absorbance values collected at 500nm. The values are mean ± SEM ($n = 4$–8 for all groups) and have been reproduced in independent experiments, $^{*}p < 0.05$ (nd = not determined).

3.5. Effect of anti-PA antibody on RAW264.7 cell death

Because of the well-described effects of PA in the sensitivity or resistance of Mϕs, we sought to determine the role of PA in RAW264.7 cell death when co-cultured with V1B. Under identical culture condition as described above, RAW264.7 Mϕs were co-cultured with V1B and anti-PA antibody was added at 10 or 20 μg/ml every 2h. As shown in Fig. 4, a dose-dependent increase in NR activity was observed in RAW264.7 Mϕs such that NR activity in treatment Mϕs following the addition of 20 μg/ml anti-PA was comparable to control Mϕs at 7.5h.

4. Discussion

Defining the mechanisms responsible for the differential susceptibility of the Mϕ cell lines IC-21, RAW264.7, and J774A.1 to purified LeTx (Kim et al., 2003; Singh et al., 1989) may be critical in understanding the pathogenesis of anthrax and treating the disease. Importantly, the toxicity of LeTx appears to depend on other factors produced by BA (Pickering and Merkel, 2004; Erwin et al., 2001; Dixon et al., 2000; Guidi-Rontani et al., 1999; Pezard et al., 1991). Therefore, developing models that examine Mϕ differential susceptibility to live LeTx-producing BA will better define the activity of LeTx in a more physiologically relevant system. The objective of the present work was to determine if IC-21, RAW264.7, and J774A.1 Mϕs would also demonstrate differential susceptibility when co-cultured with a $LeTx^{+}$ V1B strain of BA. Cytotoxicity was evaluated using morphology, TB staining, NR activity, and LDH activity in the culture supernatant. The data presented suggests RAW264.7 and J77A.1 Mϕs demonstrate signs of cytotoxicity at 7.5 and 10h, respectively, following the addition of V1B spores. In contrast, IC-21 Mϕs

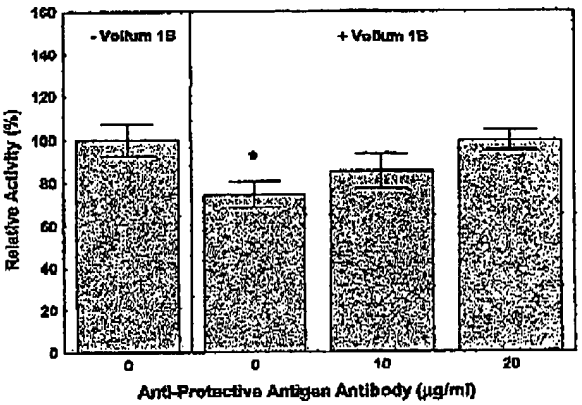

Fig. 4. NR activity in RAW264.7 Mϕs co-cultured with V1B and anti-PA antibody at 7.5h. Mϕs were cultured either in media alone (–Vollum 1B) or with V1B (+Vollum 1B) with or without anti-PA antibody (10 or 20 µg/ml). Similar to observations presented in Fig. 3B, V1B caused a decrease in NR activity in RAW264.7 Mϕs, which was dose-dependently reversed with the addition of anti-PA antibody. Data are mean ± SEM ($n = 4$ for all groups), $^*p < 0.05$ using a one-way ANOVA with LSD post hoc analysis.

remained viable and activated for greater than 15 h following the addition of V1B spores. Furthermore, the addition of anti-PA antibody to the RAW264.7/V1B culture appeared to dose-dependently protect RAW264.7 cell death. Collectively, these results suggest that Mϕ cell lines with differential susceptibility to purified LeTx also demonstrate differential susceptibility when co-cultured with a $LeTx^+$ strain of BA.

The mechanism of IC-21 resistance to V1B spores in the present work remains to be defined, but may be due to an inability of IC-21 Mϕs to generate reactive oxygen intermediates (ROIs) in response to LeTx. During the respiratory burst, Mϕs can produce large concentrations of microbicidal superoxide anion (O_2^-), hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2), and hypochlorate (^-OCl) through the NADPH oxidase complex (Ismail et al., 2002). These non-specific microbicidal agents, if produced in high concentration through hyperstimulation of the NADPH complex, can be toxic to the cell producing them. IC-21 Mϕs lack the ability to produce ROIs associated with the respiratory burst (Scott et al., 1985) and are resistant to the LeTx-induced cytotoxicity, suggesting the respiratory burst may be involved in Mϕ sensitivity or resistance to purified LeTx (Hanna et al., 1994). Although the present work did not measure LeTx concentration in the cultures, V1B germinated when co-cultured with IC-21 cells (Fig. 1B) and germination in Mϕs is closely linked to toxin production (Guidi-Rontani, 2002). Furthermore, data presented here suggest V1B produces toxins when co-cultured with RAW264.7 cells using this culture system, as evident by cell death, and it is therefore likely that toxin production also occurs in the IC-21 Mϕ culture. Thus, it is reasonable to suggest that V1B produces LeTx when co-cultured with all three Mϕ cell lines. An important finding in this work was that IC-21 Mϕs appeared to remain resistant to a strain of BA that secretes LeTx, even in the presence of other BA-toxins that have been shown to enhance the activity of LeTx. Further tests are required to better define the mechanism of IC-21 resistance and are ongoing in our lab.

An increase in LDH activity in IC-21 Mϕ culture supernatant was detected at 10 and 15 h following the addition of BA spores (Table 1), suggesting these cells were necrotic at these time points. In contrast, cellular morphology of IC-21 Mϕs at 10 and 15 h demonstrated cellular migration (Fig. 1B and D), 98% viability using TB staining (Figs. 1D and 2), and a significant increase in NR uptake (Fig. 3A), which collectively suggest Mϕs were viable and activated throughout 15 h. This discrepancy may be explained by altered pyruvate metabolism when IC-21 Mϕs were exposed to V1B, compared to control IC-21 Mϕs. Altered pyruvate metabolism has been demonstrated in a variety of Mϕ culture conditions (Cazin et al., 1990; Bar-Eli et al., 1981), including responses to bacterial infections (Sansonetti and Mournier, 1987). Additionally, the culture media (RPMI 1640 media, ATCC) for IC-21 treatment and control Mϕs contained 0.110 g/l sodium pyruvate when added to the culture at the onset. However, over the course of the experiment, IC-21 Mϕs likely required higher energy due to their activation (based on morphology and NR activity) and therefore consumed more pyruvate during the co-culture compared to IC-21 control Mϕs, which were at rest. Thus, at 10 and 15 h, cultures with IC-21 Mϕs and V1B likely contained less pyruvate in the media, which would translate to a higher LDH value during the analysis (Table 1) and give a false positive.

LeTx is one of the main virulence factors in BA (Hanna, 1999). It has been shown that Mϕ cell lines like those used in the present work, as well as primary Mϕs, demonstrate differential susceptibility to purified LeTx (Hanna et al., 1993, 1994; Friedlander et al., 1993; Singh et al., 1989). As shown in Fig. 4, the addition of anti-PA antibody dose-dependently reversed the NR activity in RAW264.7 Mϕs co-cultured with V1B such that there was no observable difference between treatment and control RAW264.7 Mϕs with the addition of 20 µg/ml antibody every 2 h. These results have been independently reproduced using V1B bacteria that were obtained from a different source and LDH activity as the measure of cytotoxicity (data not shown). Thus, the mechanism by which V1B kills RAW264.7 cells likely involves LeTx, which may be enhanced by the presence of EdTx. Studies that examine the differential response of RAW264.7 Mϕs to V1B ($pXO1^+$) and V1B ($pXO1^-$, which lacks LeTx) will better define this observation and should be the focus of future research.

The results in the present work suggest that IC-21 Mϕs survive V1B infection while the bacteria kill RAW264.7 and J774A.1 Mϕs. This in vitro model may be useful in examining differential responses of Mϕs to BA in an attempt to better define host–pathogen interactions. However, it is unknown if these responses occur in vivo. Inbred mice differ in their resistance/sensitivity to infection with BA as well as to purified anthrax such that BALB/c mice (source of sensitive RAW264.7 and J774A.1 Mϕs) appear more sensitive than C57/Bl6 mice (source of resistant IC-21 macrophages) (Moayeri et al., 2003; Welkos et al., 1986, 1989). However, the difference may be not be due to a genetic-based sensitivity or susceptibility to lethality, rather these strains of mice demonstrate differences in time-to-death, which is also highly dependent on the strain of BA. Likewise, the differential susceptibility of Mϕ cell lines shown in the current work, and specifically the resistance of IC-21 Mϕs, may similarly reflect time-to-death differences. It is possible that V1B can kill IC-21 Mϕs if co-cultured for longer than 24 h. However, death of cell lines that are in a highly activated state for >24 h may be the result of depleting the media of essential nutrients, making interpretation of the results difficult. Furthermore, as shown in Fig. 1B, there is sufficient vegetative V1B in the IC-21 Mϕ cultures such that it is difficult to focus on the Mϕs adhered to the bottom of the culture well. As such, the high titer of vegetative V1B consume essential nutrients from the media and overrun the culture volume, further confounding interpretation of IC-21 Mϕ death at extended culture times. Determining the mechanism of IC-21 resistance to V1B, compared to sensitive Mϕ cell lines, will help to determine the role of time-to-death in this model and these studies are currently underway in our lab. Finally, using this in vitro model to examine the mechanisms associated with IC-21 resistance to live LeTx-producing bacteria may lead to treatments that render susceptible Mϕs resistant to BA in vivo.

In addition to causing necrosis, LeTx and BA spores can induce apoptosis of macrophages (Parker et al., 2002; Popov et al., 2002). The current work used toxicity assays that are associated with necrotic events and no experiments were specifically conducted to determine if apoptosis of Mϕ cell lines occurred. It is unknown if V1B spores cause apoptosis in RAW264.7 or J774A.1 Mϕs.

Anti-PA antibody appears to protect RAW264.7 Mϕ death when added over the course of the experiment. Since the only known function of PA (B-domain virulence factor) is to deliver either LF or EF (A-domain virulence factor, enzymatic activity) to the cytosol, it is likely that LF and/or EF is causing RAW264.7 cell death. Studies designed to prevent RAW264.7 death by using anti-LF antibody have not been successful to date (data not shown). Likewise, considering EdTx is thought to enhance the actions of LeTx (Pezard et al., 1991), future studies are also focused on the role of EF in V1B-induced RAW264.7 Mϕ death.

This work demonstrated that Mϕ cell lines with differential susceptibility to purified LeTx (IC-21 resistance; RAW264.7 and J774A.1 sensitivity) also demonstrate differential susceptibility when co-cultured with the V1B ($LeTx^+$) strain of BA. As with purified toxin, IC-21 Mϕs appeared resistant whereas both RAW264.7 and J774A.1 Mϕs appeared sensitive. For RAW264.7 Mϕs, death was prevented by anti-PA antibody. This culture system may provide a more physiologically relevant model to further elucidate the interactions between sensitive/resistant Mϕs and virulent strains of BA and should be further characterized. Future work should examine (i) the role of cytokines in this model, in particular the pro-inflammatory cytokines TNF-α an IL-1, (ii) the ability of other anti-PA monoclonal antibodies to prevent RAW264.7 Mϕ cell death, such as antibodies derived from experimental vaccines, and (iii) pharmacological treatments that make sensitive Mϕs resistant.

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